Technical Handbook
3rd Edition

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Biomagnetic Techniques in Molecular Biology

- mRNA Isolation
- DNA & RNA Hybridisation
- Protein and gene regulation
- Solid-phase sequencing
- PCR-ready DNA

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For mRNA quality determination using agarose gel electrophoresis, elute and load 10% of one isolation (estimated to yield 10 µg mRNA) in a small well in a 1% agarose gel used for normal DNA separation (without formamide). Add cold RNase-free loading buffer to the mRNA and load it quickly into the well. Less than 0.2 µg mRNA can be detected on a 1% mRNA agarose gel if loaded into a small well and the gel run for a short period. Sensitivity can be increased by staining the gel with SYBR™ Green II Nucleic Acid Gel Stain instead of ethidium bromide. The mRNA should appear as a faint smear downwards from approximately 10 kb depending on the tissue (commonly between 0.5 and 4 kb).

Only trace rRNA bands should be visible. The presence of traces of rRNA should not affect the functionality of the isolated mRNA for most applications. For applications that require very high purity mRNA, please refer to the technical tips on reducing rRNA contamination (section 6.6).

6.8 Elution of mRNA in formamide for Northern blot analysis

Both absorbence measurements and visualisation of mRNA on a gel are relatively crude techniques for assessing the quality of mRNA. By performing a Northern blot and hybridisation of a probe directed towards the 5'-end of a mRNA with a moderate to abundant expression, the quality of the isolated mRNA can be assessed with greater accuracy.

For standard Northern blot analysis, the mRNA may be eluted directly in a loading buffer containing formamide and then loaded onto the gel. For detection of rare mRNAs, between 0.5 and 3.0 µg of poly A^+ mRNA should be applied to each lane of the gel, as compared to typically 10-30 µg when working with total RNA.

If Northern blot gives a poor signal for the larger mRNAs (> 3kb), one explanation may be the capacity of the Dynabeads and the size distribution of the isolated mRNA. The smaller molecules tend to diffuse faster, and will bind preferentially to the Dynabeads Oligo $(dT)_{25}$. By using more Dynabeads Oligo $(dT)_{25}$ or re-extracting from the lysate, the yield of larger fragments will be increased (see also figure 8, page 45).

7. Construction of immobilised cDNA libraries for multiple RT-PCR amplifications

By using Dynabeads and magnetic separation technology, pure, intact poly A^+ mRNA can be obtained either from total RNA preparations or directly from lysates of solid tissues and cell lines, as described in the preceding sections. One advantage of this approach is that it is not necessary to elute the captured mRNAs from the Dynabeads for construction of a cDNA library (51, 84, 85, 102) and the presence of the Dynabeads has no inhibitory effect on downstream enzymatic applications (see Figure 8). Solid-phase cDNA libraries specific for a particular cell type or tissue can be created directly on the surface of the Dynabeads (e.g. 2, 51, 59, 61, 85, 88, 98, 102).

The following protocols for cDNA synthesis have been devised for use with the Dynabeads mRNA DIRECT™ kit, but may also be used with the Dynabeads mRNA Purification kit or with other procedures for mRNA

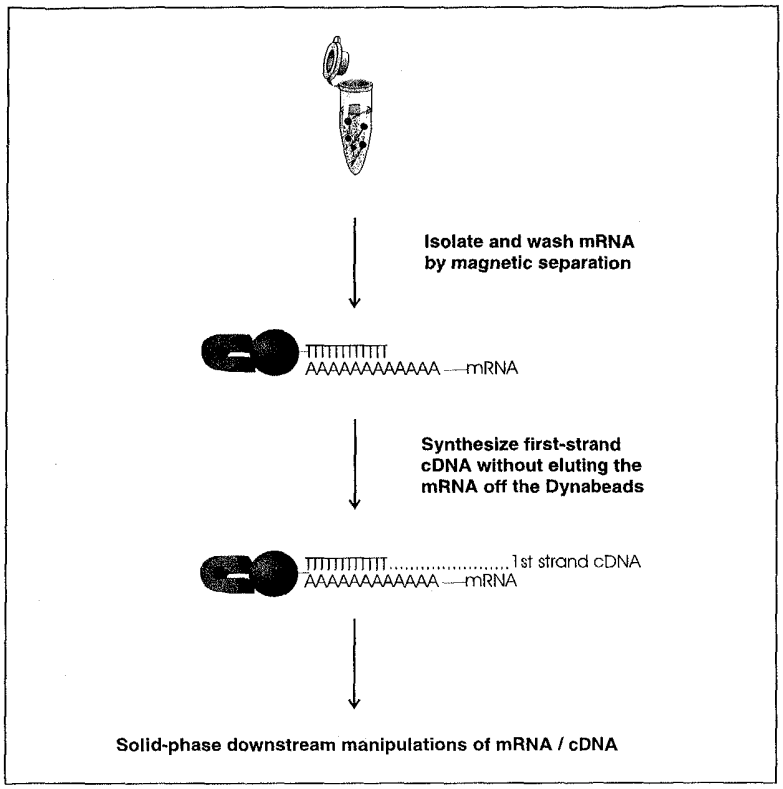

Figure 7. *Schematic flow-diagram for RT-PCR/construction of cDNA libraries and downstream manipulations using Dynabeads.*

isolation using Dynabeads Oligo $(dT)_{25}$. The protocols can also be customised for small to large scale cDNA synthesis (see Table 10 below)

Table 10: Recommended volumes and magnet stands for small to large scale cDNA synthesis.

	Small scale	Medium scale	Large scale
Dynabeads Oligo $(dT)_{25}$	10 µl = 50 µg	60 µl = 300 µg	250 µl = 1250 µg
Cells	up to 150000	up to 1 million	up to 4 million
Recommended no. of cells	80000 - 100000	500000 - 750000	2 - 3 million
Max. volume mRNA isolation	250 µl	500 µl	1 µl
Max. volume cDNA synthesis	20 µl	50 µl	200 µl
Recommended tube	Microtube/ Microamp	Microtube	Microtube
Recommended magnet stands	Dynal MPC-P-12 Dynal MPC-9600	Dynal MPC-P-12 Dynal MPC-E	Dynal MPC-E Dynal MPC-M

The solid-phase synthesis of a cDNA library using Dynabeads Oligo $(dT)_{25}$ ensures that washing to remove detergent (LiDS) and salts before the

Figure 8. PCR-an cation is not inhi by Dynabeads. *D isolation of mRNA 2 million Daudi-ce (cultured B-cell lin using 200 µl (1 mg Dynabeads Oligo followed by solid- cDNA synthesis u SuperScript™ II a ding to the supplie instructions. After removal of the mF the immobilised c on Dynabeads wa amplified in a 50 µ upstream of the p used in each amp (lanes 9-10), 200 µ negative control v*

7.1 Advan

enzymatic step can be conducted simply and effectively. All reverse transcriptases which have been tested have been found to function in solid-phase cDNA synthesis (AMV, M-MLV, SuperScript™, rTth, Retrotherm™). The size of the RT-PCR product will not be limited by the mRNA isolation, but by the RT and PCR enzymes used and their functionality (see Figure 8). The results in this figure illustrate both that the PCR is not inhibited by the presence of the Dynabeads and also that the cDNA synthesis is not limited by the mRNA isolation.

The field of RT-PCR is the focus of much active research and is changing rapidly as techniques develop and knowledge is accrued. The following protocols should be regarded as suggestions made in the light of our present knowledge. Always follow the instructions given by kit/enzyme suppliers.

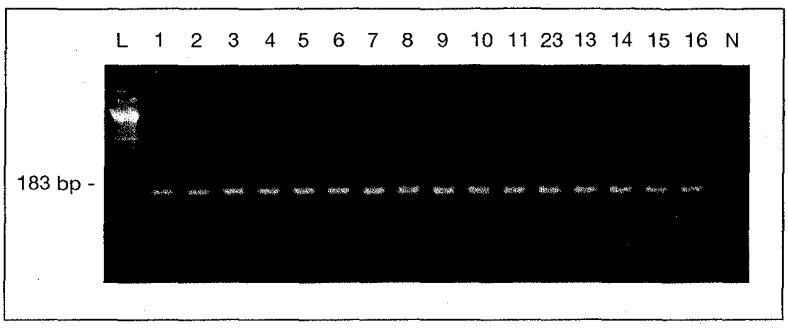

Figure 8. PCR-amplification is not inhibited by Dynabeads. *Direct isolation of mRNA from 2 million Daudi-cells (cultured B-cell line) using 200 µl (1 mg) Dynabeads Oligo (dT)$_{25}$ followed by solid-phase cDNA synthesis using SuperScript™ II according to the supplier's instructions. After removal of the mRNA, the immobilised cDNA on Dynabeads was amplified in a 50 µl PCR volume using PSK-H1 specific primers. The resulting 183 bp amplicon lies 4.3 kb upstream of the poly A-tail in the PSK-H1 transcript. The different amounts of solid-phase cDNA on Dynabeads used in each amplification was 10 µl (lanes 1-2), 25 µl (lanes 3-4), 50 µl (lanes 5-6), 100 µl (lanes 7-8), 150 µl (lanes 9-10), 200 µl (lanes 11-12), 250 µl (lanes 13-14) and 500 µl (lanes 15-16). Lane L: 100 bp ladder. Lane N: negative control without RT-template.*

7.1 Advantages of using Dynabeads® for immobilised cDNA libraries

Following capture of mRNA by Dynabeads Oligo (dT)$_{25}$, the Dynabeads-bound mRNA can also be used as a primer for reverse transcriptase to synthesise first-strand cDNA, thus creating a covalently-linked first-strand cDNA library. The construction of a reusable solid-phase cDNA library allows multiple downstream amplifications of specific transcripts. The second-strand cDNA is synthesised using the specific primer. This second-strand is melted off from the solid-phase and can be used as a template for PCR amplification (Figure 10). The solid-phase cDNA library, recovered by magnetic separation from the supernatant, can be reused for several specific PCR amplifications.

The major advantages of using Dynabeads for construction of immobilised cDNA libraries are listed below.

- The system is well suited for extraction of small quantities of mRNA and one extraction allows the amplification of several genes (31).
- The Dynabeads unique 'magnetic handling' property enables simple and rapid buffer changes required to optimise the conditions for specific enzymes.

- All reactions can be conducted in a single tube and losses at each step are minimised (Figure 12).
- Simple, effective washing following synthesis of the first-strand cDNA minimises contaminants (e.g. free oligo (dT) primers, enzymes, buffer differences) in the reverse transcriptase reaction. Contaminants are likely to cause background problems, generating smears on agarose gels. A low background simplifies cloning (3).
- mRNA isolation and subsequent cDNA synthesis using a thermostable enzyme can be conducted in under one hour, without loss of mRNA material.
- Multiple rounds of the PCR reaction can be conducted directly on the solid-phase cDNA and the Dynabeads themselves cause no inhibition of the reaction (Figures 8 and 11).
- Only small amounts of cDNA Dynabeads are necessary for cDNA amplification and there may be no need for reuse of these beads. It is then possible to move directly from first-strand cDNA synthesis to a PCR amplification with the beads present during the cycling reactions.
- The identification and amplification of specific cDNA molecules for downstream analysis and applications is simplified.
- The solid-phase provides convenient cDNA storage.
- The solid-phase cDNA libraries are reusable (Figure 10).
- The system provides potential for wide-ranging and far-reaching studies, for example cell- or stage-specific gene expression studies.
- The simplicity and minimal losses inherent in the technique enable amplification of transcripts from single cell samples (see below and 54).

Whilst RT-PCR has been proven sensitive enough to detect transcripts from a few or even a single cell, the traditional techniques typically require lysis of cells in the presence of high concentrations of guanidinium thiocyanate (GTC) and/or the purification of the RNA by caesium chloride ultra-centrifugation and/or extraction and precipitation of the RNA. These steps are not only time-consuming and cumbersome, but may result in some loss of the RNA. By purifying mRNA with Dynabeads Oligo $(dT)_{25}$, RT-PCR has successfully been used to amplify transcripts from single cell samples (54 and Figure 9 below). In the work reported by Karrer et al (54), the contents of individual cells were aspirated into micro-pipettes filled with RNA extraction buffer and cDNA synthesis was conducted with SuperScript™ II reverse transcriptase for 60 minutes at 45°C. RT-PCR detection was possible on Dynabeads captured mRNA. Losses were minimised at each purification step as all the operative manipulations occur on the solid-phase.

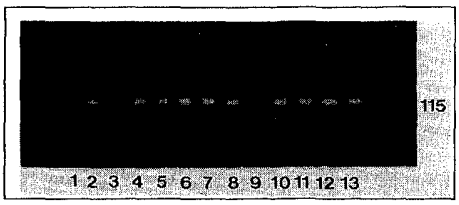

Figure 9. RT-PCR on mRNA isolated from minute amounts of sample. *Amplification using β-microglobulin specific primers on mRNA isolated from Reh-cells (pre-B cell line) resulting in a 115 bp fragment. Lane 1: 100 cells with RNase treatment prior to RT-PCR; lane 2: 100 cells; lane 3: negative control; lanes 4-13: mRNA isolated from on average 1.8 cells. Only lane 9 is negative. The probability of getting one negative reaction is less than 0.02, if single cells are not detectable. Courtesy of A. Deggerdal, The Norwegian Radium Hospital, Norway.*

7.2 The principle of immobilised cDNA libraries

The Oligo (dT)-sequence bound to the Dynabeads Oligo $(dT)_{25}$ surface is used to capture mRNA. In the construction of immobilised cDNA libraries, this Oligo (dT)-sequence is also used as a primer for the reverse transcriptase to synthesise the first-strand cDNA (see Figure 7). This results in a covalently-linked first-strand cDNA library that can be used for specific applications such as cDNA amplification (51, 59, 61, 84, 85, 102), cDNA cloning (59) or subtractive hybridisation (2, 19, 88, 98, 102).

RACE PCR (rapid amplification of cDNA ends) has proven to be a valuable tool for obtaining the ends of gene transcripts and can be usefully combined with using Dynabeads to provide a solid-phase support. Using Dynabeads, a solid-phase cDNA library for RACE was constructed by dG tailing of the cDNAs (61). 5' RACE was performed by amplification with a 3' gene specific primer and a 5' adapter poly(dC) primer. For the 3' end amplification a 5' end specific primer and a 3' poly(dT) primer were used. The amplicons were separated from the cDNA Dynabeads using a magnet stand and the recovered dG-tailed cDNA Dynabeads could be reused for at least five rounds of PCR (61). By using one biotinylated primer in the amplification, the amplicons can subsequently be sequenced by standard solid-phase protocol (Chapter 4, section 2).

Construction of a cDNA library from tiny amounts of mRNA by PCR-amplification of cDNA that has been tailed at the 3' end of the first-strand cDNA has been a successful strategy. By synthesising an immobilised cDNA library on Dynabeads all manipulations can be carried out in one tube. Difficulties such as yield loss due to multiple transfers and precipitations associated with other protocols are avoided. For example, a representative library has been cloned from about 5 ng polyadenylated RNA from tomato root tips (59). After cDNA synthesis, unprimed oligo $(dT)_{25}$ on the Dynabeads were removed by T4 DNA polymerase before the cDNA strands were A-tailed by terminal transferase. The second cDNA strands were synthesised with an Oligo (dT) primer with a tail sequence. These second-strand cDNAs were released, amplified and subsequently cloned.

How to deal with DNA contamination

Using a direct mRNA isolation method incurs the risk of DNA contamination. Whilst this is usually not a problem with the Dynabeads mRNA DIRECT™ system, it may occur when larger amounts of material are used or the tissue is inherently difficult (e.g. spleen). It is recommended to start with a maximum of 1 million cells in a 1 ml isolation volume (Lysis/Binding buffer) in order to isolate pure mRNA for cDNA synthesis. It is also important to reduce the viscosity either by diluting the sample or by DNA-shearing as described in section 6.5. Another option is to purify the mRNA twice using Dynabeads Oligo $(dT)_{25}$ and follow the standard protocol as described in section 6.6.

To ensure that it is cDNA, and not genomic DNA, which is detected, various controls are possible.

a. Choose primers in neighbouring exons to obtain different amplicon sizes for cDNA and genomic DNA (e.g. as in Figure 11 for PSK-H1).
b. Include a negative control with reverse transcriptase omitted.
c. Use genomic DNA-specific primers from non-transcribed regions to identify contamination by resulting in PCR products of different sizes when compared to that from RT-PCR amplification.

d. Remove DNA contamination by using RNase-free DNase to treat the mRNA sample before cDNA synthesis.

7.3 Materials required (including buffers)

- Dynabeads Oligo $(dT)_{25}$ (Prod. No. 610.02/05)
- Washing buffer with and without LiDS (see section 4)
- RT Buffer for specific enzymes (e.g. AMV, M-MLV, SuperScript™, *rTth*, Retrotherm™)
- RT-mix with nucleotides
- Reverse transcriptase (e.g. AMV, M-MLV, SuperScript™, *rTth*, Retrotherm™)
- TE Buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA)
- Elution solution (10 mM Tris, pH 7.5)
- EZ-rTth mix (as described in the Perkin Elmer GeneAmp® kit)
- Magnet stand - Dynal MPC (see Chapter 1, section 3)
- Water bath
- Programmable heat-block
- Sterile, RNase-free microtubes of Eppendorf type
- Sterile, RNase-free pipettes and pipette tips
- Deionized, RNase-free water
- Diethyl pyrocarbonate (DEPC)

7.4 Protocols for cDNA synthesis

7.4.1 Synthesis of a reusable solid-phase cDNA library

1. Capture mRNA with Dynabeads Oligo $(dT)_{25}$ either directly from cell lysates or from total RNA preparations as previously described (section 5). Titrate the number of cells and amount of Dynabeads used. When working with proliferating cells 10 µl Dynabeads (approximately 50 µg) is recommended for approximately 100,000 cells.
   Note: The numbers given are for medium scale libraries; numbers for small scale are in square brackets (see also Table 10, page 44).
2. Wash mRNA/Dynabeads Oligo $(dT)_{25}$ complexes twice with Washing buffer with LiDS and once with Washing buffer as described in section 5, but do not elute mRNA from the Dynabeads.
3. To ensure complete removal of LiDS and LiCl, wash the mRNA/ Dynabeads Oligo $(dT)_{25}$ complexes three times in 250 µl cold RT-buffer [50 µl]. Transfer to a new RNase-free tube between second and third wash.
4. Remove RT-buffer after the third washing step using the magnet and resuspend mRNA/ Dynabeads Oligo $(dT)_{25}$ complexes in RT-mix with nucleotides, buffer and enzyme.
   Note: Use a maximum of 300 µg Dynabeads [50 µg] per cDNA synthesis in a volume of 50 µl [20 µl].
5. Perform cDNA synthesis as recommended by the manufacturer of the RT enzyme.
   Note: Any reverse transcriptase can be used, but heat-stable enzymes like Retrotherm™ and rTth are useful as elevated temperatures will reduce mRNA secondary structure. Use buffers and conditions as specified with the enzyme protocol. A combination of enzymes may be used; cDNA synthesis at 37-42°C for 45 minutes, a rapid change of buffer by using a magnet stand, and incubation at 72°C for 10 minutes with the

Figure 10. *Sch flow diagram fo struction of a re solid-phase cDN and downstrean amplification.*

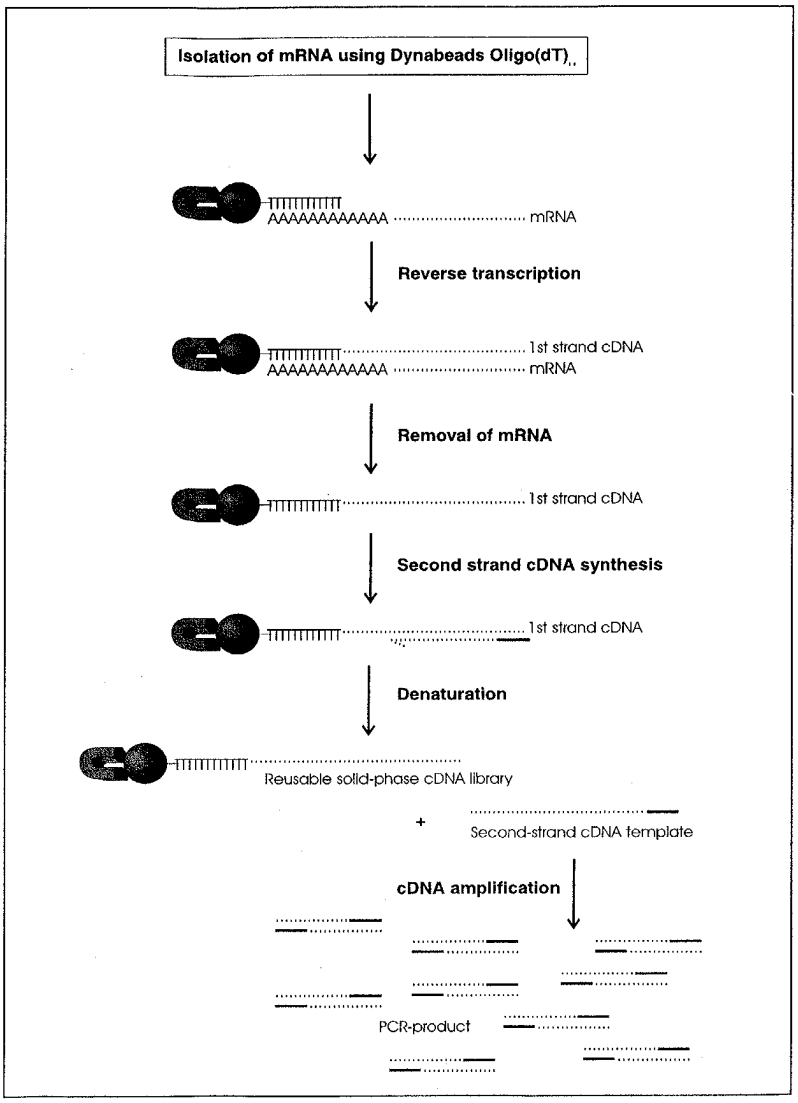

Figure 10. *Schematic flow diagram for construction of a reusable solid-phase cDNA library and downstream RT-PCR amplification.*

thermostable enzyme. The synthesis can be done using a heating block, a water bath or a thermocycler. If possible, the tube should be mixed gently every 10 minutes to keep the Dynabeads in suspension to improve the cDNA synthesis. The use of a hybridisation oven to obtain constant rolling and the temperature for optimal cDNA synthesis is recommended.

6. After first-strand cDNA synthesis is completed, collect Dynabeads using a magnet stand and remove the RT-mix.
7. Resuspend Dynabeads in 50 µl of Elution solution and heat to 95°C for 1 minute.
8. Immediately collect Dynabeads using a magnet stand, and remove supernatant containing melted mRNA.
9. Wash cDNA/Dynabeads once in TE-buffer and store in this buffer at

4°C. It is also possible to wash and store the solid-phase cDNA library in SSC, 70% ethanol or water. The single-stranded cDNA library is stable in this form in nuclease-free solutions.

7.4.2 PCR-amplification from a reusable solid-phase cDNA library

1. Remove storage solution from the solid-phase cDNA library using a magnet stand and wash the cDNA/Dynabeads once in the enzymatic buffer to be used.
2. Resuspend the cDNA/Dynabeads in PCR-mix. Use 20 - 50 µg or a maximum of 100 µg cDNA/Dynabeads per amplification.
3. Cycle once to make 2nd strand cDNA with extension at 72°C.
4. Melt strands at 94°C for 2 minutes.
5. Place tube in a magnet stand and transfer supernatant with second-strand cDNA to a new PCR tube.
6. Use this second-strand cDNA as template for PCR-amplification.
7. Wash cDNA/Dynabeads once in TE-buffer and store in this buffer at 4°C. It is also possible to wash and store the solid-phase cDNA library in SSC, 70% ethanol or water. The single-stranded cDNA library is stable in this form in nuclease-free solutions.

   Note: If it is not necessary to reuse the Dynabeads, the PCR cycling reactions can be run with the Dynabeads present (see section 7.4.3 below).

   Note: Subsequent solid-phase sequencing of the amplified cDNA is possible if one biotinylated primer and one non-biotinylated primer is used in the PCR reaction. Remove any Dynabeads Oligo $(dT)_{25}$, then bind the biotinylated PCR-product to Dynabeads Streptavidin. Proceed with making a single-stranded sequencing template as described in Chapter 4, section 2.

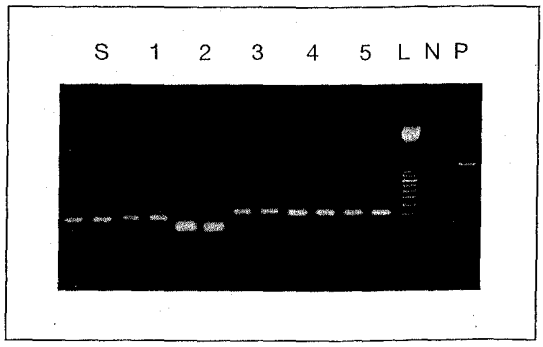

Figure 11. Reuse of a solid-phase cDNA library for RT-PCR amplification. *20 µl Dynabeads Oligo $(dT)_{25}$ was used to isolate mRNA directly from 300,000 cultured HL-60-cells, followed by 1st strand cDNA synthesis using the Dynabeads-bound Oligo $(dT)_{25}$ as primer. One half of the resulting solid-phase cDNA library was used as template for full PCR-amplification of GAPDH (S). For the other half, the amplicon was separated from the Dynabeads after 3 PCR-cycles and used as template for PCR-amplification of GAPDH (1). This solid-phase cDNA library was then washed and reused four times for amplification of PSK-H1 (2), β-actin (3), RING11 (4), and CD19 (5). L = 100 bp ladder, P = positive control; PSK-H1-amplification of genomic DNA, N = negative control without RT-enzyme; no DNA contamination detected. (69).*

7.4.3 RT-PCR amplification from a solid-phase cDNA library with Dynabeads present in the PCR.

If reuse of the beads is not required, the PCR can be run with the beads present through the sequence of cycling reactions. The Dynabeads will not affect the PCR (see Figure 8). Also, cDNA/Dynabeads complexes

Figure 12. One-tul RT-PCR using Dynabeads Oligo (*Direct isolation of m from 150,000 Daud (cultured B-cell line, 10 µl (50 µg) Dynab Oligo $(dT)_{25}$ per lane Solid-phase cDNA synthesis with rTth merase for 5 min at and 25 min at 61°C, directly followed by PCR-cycles to amp the different transcr lane 1: GAPDH (266 PCR-controls (as 1- resulting in amplico results in GAPDH-p genomic DNA as sh*

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